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Human Cytomegalovirus US28: A Functionally Selective Chemokine Binding Receptor

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Abstract

The Human Cytomegalovirus (HCMV)-encoded chemokine receptor US28 is the most well-characterized of the four chemokine receptor-like molecules found in the HCMV genome. US28 been studied as an important virulence factor for HCMV-mediated vascular disease and, more recently, in models of HCMV-associated malignancy. US28 is a rare multi-chemokine family binding receptor with the ability to bind ligands from two distinct chemokine classes. Ligand binding to US28 activates cell-type and ligand-specific signaling pathways leading to cellular migration, an example receptor functional selectivity. Additionally, US28 has been demonstrated to constitutively activate PLC and NFkB. Understanding the structure/function relationships between US28, its ligands and intracellular signaling molecules will provide essential clues for effective pharmacological targeting this multifunctional chemokine receptor.

Introduction

The ubiquitous β-herpesvirus, HCMV establishes a life-long persistent/latent infection in the immunocompetent host. Although HCMV infections are largely asymptomatic in individuals with normal immune function, HCMV has been implicated in the development of vascular diseases including transplant vascular sclerosis associated with chronic rejection of transplanted solid organs, restenosis following angioplasty and atherosclerosis (Hendrix et al., 1989; McDonald et al., 1989; Melnick, Adam, and DeBakery, 1998; Melnick et al., 1983; Muhlestein et al., 2000; Speir et al., 1994; Zhou et al., 1996). HCMV infection is also associated with malignancies (Cobbs et al., 2002; Harkins et al., 2002; Scheurer et al., 2008; Soderberg-Naucler, 2006) but the mechanisms of cytomegalovirus contribution to cancer remains poorly understood (Soderberg-Naucler, 2006) and is more likely oncomodulatory rather than oncogenic in nature (Cinatl et al., 2004).

HCMV encodes four chemokine receptor homologues, namely UL33, US27, US28 and UL78 (Chee et al., 1990). Of these, US28 is the most highly characterized. US28 has been established as a key mediator of HCMV-associated vascular disease (Streblow, Orloff, and Nelson, 2001) and has recently been implicated in models of HCMV-associated glioblastoma (Maussang et al., 2006). US28 has been extensively studied as a consitutive activator of phospholipase C and NF-kB (Casarosa et al., 2001). However, a number of ligand-dependent US28 activities have been characterized including the initiation of calcium flux (Gao and Murphy, 1994; Kuhn, 1995) and the activation of MAP kinase signaling pathways (Billstrom et al., 1998) as well as directed chemotaxis of vascular smooth muscle cells and macrophages (Streblow et al., 1999).

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Characterized US28 ligands include a number of human CC-chemokines (RANTES, MCP-1, MCP-3 and MIP-1α) as well as the CX3C-chemokine Fractalkine. US28 is unusual among chemokine receptors in its ability to bind chemokines of distinct chemokine families. The functional implications of this unique property of US28 are just beginning to be understood, and indicate that US28 displays "functional selectivity" defined as the ability of a single receptor to activate different signaling pathways in a ligand-specific manner (Urban et al., 2007). This extended understanding of US28 signaling has significant implications for rational drug design to target the various functions of US28 in the context of HCMV pathogenesis and, as such, is the major focus of this review.

Chemokine Receptor Structure and Function

General

Chemokine receptors (CCRs) comprise a subfamily of the large Class A (Rhodopsin-like) 7-transmembrane domain (7-TM) G protein-coupled receptors (GPCRs). CCRs share many characteristics with other GPCRs but contain several common characteristics by which they can be distinguished. CCRs are generally 340-370 amino acids (aa) in length and contain both acidic residues and sites for tyrosine sulfation in their N-termini. A cystine is often found in each of the four extracellular domains. There is a conserved DRY-motif found in the second intracellular loop and the third intracellular loop contains basic residues (Murphy et al., 2000). Although no crystal structure exists for any CCR, their structure has been inferred based upon sequence similarity to rhodopsin (Baldwin, 1993; Lomize, Pogozheva, and Mosberg, 1999; Unger et al., 1997). However, the synthesis of numerous functional and mutagenesis studies have provided a toggle-switch model for GPCR activation in which TM-3, TM-6 and TM-7 move towards each other on the extracellular face and away from each other on the intracellular face, utilizing proline residues as pivot points. The spreading of these three helices on the intracellular face of the receptor allows for the binding of signaling proteins such as G-proteins and subsequent intracellular signal transduction. This model attempts to reconcile the structural similarity of GPCRs and the commonality of their intracellular effector molecules (i.e. G-proteins and arrestins) with the vast diversity of GPCR agonists, which range from metal ions to small molecules to large glycoprotiens. The model is based upon the assumption that agonists function to stabilize, rather than induce, the activated receptor conformation. Therefore, diverse ligands can interact with different residues in distinct portions of the receptor to affect this stabilization. Interestingly, this model also provides for the possibility of spontaneous adoption of the active conformation in the absence of an agonist to stabilize, which could account for the constitutive activity observed with a number of GPCRs (Schwartz et al., 2006).

Ligand Binding

Unlike small molecule agonists that generally bind inside the hydrophilic pocket formed by the TM helices, chemokines and other peptide ligands are thought to stabilize activated receptor conformations via multiple low affinity interactions in several regions of the extracellular and TM domains. The N-terminus of chemokine receptors is often (but not always) indespensible for high affinity chemokine binding (Ahuja, Lee, and Murphy, 1996; Lau et al., 2004; Monteclaro and Charo, 1996).

G-protein Binding

The highly conserved D/ERY or 'DRY' motif at the intracellular end of TM-3 of all GPCRs has received significant attention as a potential G-protein binding and receptor activation determinant. Unfortunately, phenotypes observed with mutations in this region vary widely from receptor to receptor, even within the Class A family of GPCRs (Rovati, Capra, and Neubig, 2007). Mutation of the D/E residue often results in an increase in constitutive

activity concurrent with an increase in agonist affinity. This phenotype has been observed with CXCR2 mutants (Burger et al., 1999) and would suggest a destabilization of the inactive receptor conformation. Similar mutations in CCR3 and CX3CR1 did not result in an increase in constitutive activity (Auger et al., 2002; Haskell, Cleary, and Charo, 1999). There is a similar lack of a consensus phenotype with mutations of the conserved arginine residue (Rovati, Capra, and Neubig, 2007). The two chemokine receptors for which these mutations have been studied (CCR3 and CX3CR1) display a decrease in agonist-induced activity, most likely due to a defect in G-protein binding (Auger et al., 2002; Haskell, Cleary, and Charo, 1999). These results suggest that the conserved arginine plays a significant role in G-protein binding, however the current data are not conclusive in this regard.

Recent evidence suggests that different ligands can stabilize receptor active conformations that have a G-protein affinity bias. This agonist-specific activation of signaling via distinct G-proteins has been demonstrated for the mu-opioid receptor (Saidak et al., 2006), the beta-2 adrenergic receptor (Woo et al., 2008) and the thromboxane A2 receptor (Zhang, Brass, and Manning, 2008). Taken together, these data indicate that the cellular G-protein environment as well as the agonist environment can influence the signaling capabilities and cellular functions of GPCRs.

Desensitization and Regulation

Regulation of chemokine receptor signaling proceeds via the generally accepted model for GPCR regulation in which the ligand-stabilized, activated receptor transduces signal via G-protein coupling and is subsequently phosphorylated on the intracellular face by one or more G-protein coupled receptor kinases (GRKs). Phosphorylation mediates the coupling of arrestins, which prevent further G-protein coupling and facilitate internalization of the desensitized receptor. The receptor is then either recycled to the cell surface in a process of resensitization or targeted for degradation (Krupnick and Benovic, 1998; Pitcher, Freedman, and Lefkowitz, 1998). This “classical” model for GPCR regulation has been revised and extended recently to account for the tissue-specific (Tobin, Butcher, and Kong, 2008) and ligand-specific (Kelly, Bailey, and Henderson, 2008) control of GPCR signaling.

In addition to the seven known human GRKs, second messenger-dependent protein kinases such as PKA and PKC can phosphorylate GPCRs to facilitate desensitization (Benovic et al., 1985). Interestingly, CCR5 has been shown to be phosphorylated by both PKC and GRK 2 and/or 3 at different sites with different kinetics in a ligand-specific manner (Pollok-Kopp et al., 2003). However, the impact of these differential phosphorylation events on receptor function remains to be elucidated. These regulatory kinases differ in their tissue distribution, providing a mechanism for cell-type specific regulation of GPCR activity (Tobin, Butcher, and Kong, 2008). Furthermore, the expression of GRKs can be modulated in a specific cell type in response to pro-inflammatory stimuli. This is of particular importance in the regulation of chemokine receptor activity in hematopoetic cells and the development of various inflammatory diseases (Vroon, Heijnen, and Kavelaars, 2006). In particular, decreased GRK2 and GRK6 expression in immune cells is observed in experimental models of arthritis (Lombardi et al., 2001). Further, TLR-4 mediated signaling via LPS stimulation has been shown to decrease expression of GRK2 and GRK5 in neutrophils (Fan and Malik, 2003). Such inflammatory modulation of GRK expression could result in increased signaling from chemokine receptors normally regulated by these kinases and provide a mechanism for increased recruitment of immune cells to sites of chronic inflammation. Indeed, decreases in GRK expression have been observed to increase chemotactic responses in T-cells via CCR5 (Vroon et al., 2004a), and neutrophils via CXCR4 and CXCR2 (Fan and Malik, 2003; Vroon et al., 2004b).

One might expect that decreases in β-arrestin expression might have a similar effect on cellular chemotaxis via a decrease in receptor desensitization and a concurrent increase in pro-migratory signaling. However, β-arrestin-2 deficient T and B cells display decreased chemotactic activity despite increases in GTPase activity associated with CXCR4 (Fong et al., 2002). This data could be attributed to the β-arrestin mediated activation of pro-migratory signaling molecules including ERK1/2 (Ge et al., 2003), and p38 MAPK (Sun et al., 2002). Although very little is known about the specificity of β-arrestin recruitment to phosphorylated receptors, there is clearly the potential for crosstalk between GRK and/or second-messenger dependent kinase-mediated phosphorylation of receptors mediating differential binding of β-arrestin proteins (Violin, Ren, and Lefkowitz, 2006) and potentially modulating the signaling capacity of receptor-β-arrestin complexes (Kelly, Bailey, and Henderson, 2008; Tobin, Butcher, and Kong, 2008).

Functions of US28 and their Pathophysiological Consequences

Cellular Activation

US28 was initially characterized as being able to cause ligand-dependent calcium flux and Gα16-mediated signaling to ERK1/2 in 293 cells (Billstrom et al., 1998). Further signaling functions were elucidated in COS-7 cells where US28 is a constitutive ‘cellular activator’. In this system, US28 signals in a ligand-independent manner to both phospholipase C and NF-kB (Casarosa et al., 2001). These pathways certainly conspire to produce a cellular environment optimal for HCMV replication and NF-kB has been shown to directly transactivate the HCMV major immediate-early promoter (Boomker et al., 2006b; DeMeritt, Milford, and Yurochko, 2004). Constitutive activation of these cellular signaling pathways may play a role in CMV-mediated inflammatory diseases and possibly CMV-mediated oncogenesis (Soderberg-Naucler, 2006; Vischer, Leurs, and Smit, 2006). In addition, US28 expression has been shown to activate caspase-dependent apoptosis in a number of cell lines (Pleskoff et al., 2005).

Chemokine Scavenging

The supernatants of HCMV-infected fibroblast cultures have been shown to be deficient in a number of CC-chemokines. This has been attributed to a chemokine scavenging function of US28 in which these chemokines are actively removed from the supernatant via the constitutive recycling of US28 protein (Bodaghi et al., 1998). US28 expression in HCMV-infected fibroblasts is sufficient to appreciably decrease the monocyte chemotactic activity of the infected cell supernatants compared to supernatants from fibroblasts infected with a US28 knockout virus (Randolph-Habecker et al., 2002). However, this role of US28 as a ‘chemokine sink’ has recently been challenged by others. US28 expression in endothelial cells is insufficient to prevent static monocyte adhesion, suggesting that physiological concentrations of chemokine are too high to be effectively scavenged by US28 (Boomker et al., 2006a).

Cellular Chemotaxis

Importantly, US28 plays a role in motility of HCMV-infected cells. US28-mediated cellular migration has important implications for both HCMV dissemination and mechanisms for the development of HCMV-associated vascular disease. US28 binding to CC-chemokines causes migration of HCMV infected SMC (Streblow et al., 1999). Conversely, stimulation of US28-expressing SMC with Fractalkine can antagonize CC-chemokine mediated SMC migration. Detailed examination of the signaling cascades involved in US28-mediated SMC migration indicate that CC-chemokine binding to US28 induces the G12-dependent activation of a variety of pro-migratory factors including FAK, Src, ERK1/2, Pyk2 and RhoA (Melnychuk et al., 2004; Streblow et al., 1999; Streblow et al., 2003). Interestingly,

Fractalkine binding to US28 activates many of the same cellular second messengers seen with CC-chemokine binding, including FAK and ERK1/2. Although both chemokine classes activate FAK via US28, they do so via different G-proteins. CC-chemokine activation of FAK is G12-mediated while Fractalkine binding to US28 activates FAK via a Gq-dependent mechanism (Vomaske et. al, in press). We have recently determined that Fractalkine but not CC-chemokines can induce migration in US28-expressing macrophages (Vomaske et al, in press). Therefore, US28 utilizes both the extracellular chemokine environment and the intracellular complement of G-proteins to produce ligand and cell-type specific migration of HCMV-infected cells. Taken together, these observations suggest a dual role for US28 in the acceleration of vascular disease via (1) CC-chemokine mediated migration of SMC into the vessel intima followed by Fractalkine-mediated fixation of SMC and subsequent proliferation leading to vessel narrowing and (2) the Fractalkine-mediated deposition of US28-expressing monocytes into atherosclerotic lesions leading to the formation of foam cells.

US28 Structure and Function

Chemokine Binding Site

Although chemokine ligands interact with multiple sites on the extracellular face of chemokine receptors, the N-terminus often dictates high-affinity ligand binding (Monteclaro and Charo, 1996). A hexapeptide sequence in the N-terminus of US28 has been shown to be critical for high-affinity binding of chemokine ligands to US28 (Casarosa et al., 2005) This region is conserved between US28 and the endogenous human chemokine receptors CCR1 and CCR2 and is known to be a determinant for MCP-1 binding to CCR2 (Preobrazhensky et al., 2000). Casarosa et. al. performed extensive mutagenesis studies of this region of US28 and discovered that CC-chemokine binding and fractalkine binding require different residues within this hexapeptide region for high affinity binding. Specifically, mutation of phenylalanine at position 14 negatively affects only CC chemokine binding to US28 but retains high affinity binding of Fractalkine. In contrast, mutation of the tyrosine at position 16 of the US28 N-terminus negatively affects high affinity binding of both classes of chemokine. The authors speculate that sulfation of Y16 may play a role in chemokine binding to US28. Additionally, an aromatic amino acid seems to be required at this position for surface expression of US28 suggesting a role in folding or trafficking of the receptor (Casarosa et al., 2005).

Heterotrimeric G-protein Binding and Activation

US28 is known to couple to a variety of G-proteins alpha subunits. This G-protein promiscuity appears to provide a level of control and selectivity to US28 signaling depending upon ligand and cell-type context. The coupling of Gq/11 family members to US28 mediates the activation of phospholipase C (PLC) and NFkB in a ligand-independent manner (Casarosa et al., 2001; Waldhoer et al., 2002). In this system, Fractalkine binding to US28 abrogates constitutive activation of PLC (Casarosa et al., 2001). Further, Fractalkine binding to US28 in fibroblasts causes Gq/11-dependent signaling to focal adhesion kinase (FAK) (Vomaske et. al., in press) Taken together, these findings indicate that US28 activates Gq/11 signaling to PLC in the absence of ligand, but Fractalkine binding to US28 co-opts the receptor and utilizes a different Gq/11-dependent pathway to activate FAK. In contrast, CC-chemokine dependent US28 migration of SMC proceeds via G12/13-mediated signaling to FAK, ERK, RhoA and the actin cytoskeleton (Melnychuk et al., 2004; Streblow et al., 1999). In 293 cells, RANTES binding to US28 activates ERK1/2 pathways through the G-proteins Gαi1 and Gα16 (Billstrom et al., 1998). The highly conserved ‘DRY’ motif at the end of TM-3 is critical for US28 signaling to PLC (Waldhoer et al., 2003). In contrast, mutagenesis of this motif has no effect on US28-mediated SMC migration (Table 1).

Experiments with the OT-1 receptor showed that mutation of the DRY motif could simultaneously decouple the receptor from Gi proteins while augmenting signaling via Gq, indicating that different G-proteins may have different sequence requirement at this position and that not all G-protein coupling requires a functional DRY (Favre et al., 2005). Taken together, these results indicate that different activated forms of US28 (i.e. CC- vs. CX3C-chemokine bound or unliganded constitutively active) may utilize slightly different sites for G-protein interaction and activation contributing to G-protien and ligand binding specificity.

Regulation and Desensitization

US28 is heavily phosphorylated in a ligand-independent manner on several serine residues within the C-terminal 30 amino acids. This phosphorylation can be reduced by pharmacological inhibition of PKC and casein kinase 2 (CK2) and is enhanced by overexpression of GRK2 or GRK5 (Miller et al., 2003; Mokros et al., 2002; Sherrill and Miller, 2006). Although US28 phosphorylation in cell culture systems appears to be ligand-independent, phosphorylation levels can be modulated by US28 ligands. In HEK293 cells, Fractalkine can decrease this basal phosphorylation levels (Mokros et al., 2002). The same effect has been observed with RANTES treatment in COS7 cells (Miller et al., 2003). Taken together, these results indicate that regulatory kinases and phosphatases interact in a dynamic manner with different activated states of US28. Phosphorylated forms of US28 are known to recruit β-arrestin-2 and this association of β-arrestin with US28 can be augmented by overexpression of GRK2 (Miller et al., 2003). However, the constitutive endocytosis and recycling of US28 is not effected by expression in β-arrestin deficient cells (Fraile-Ramos et al., 2003). Truncation mutants of US28 that remove the C-terminal phosphorylation sites are generally more prevalent at the cell surface and display increased signaling to PLC, NFkB and CREB (Miller et al., 2003; Mokros et al., 2002; Waldhoer et al., 2003). However, one study which mutated the C-terminal serine residues rather than truncating the protein showed that serine phosphorylation has no effect on US28 signaling to NFkB (Mokros et al., 2002). Interestingly, while Fractalkine treatment generally decreases US28 consitutive signaling, C-terminal truncation mutants display increased accumulation of inositol phosphages and increased transcription from a CREB-responsive element when treated with Fractalkine. The authors attribute this phenotype to increased surface expression of the truncated US28 construct leading to increased binding of the ligand (Waldhoer et al., 2003). Experiments with the MCMV-encoded GPCR M33 suggest that the constitutive activity of this functional homolog of US28 is regulated both by phosphorylation by GRK2 and GRK2-mediated sequestration of Gq proteins (Sherrill and Miller, 2006). The effect of ligand binding on US28 phosphorylation via various regulatory kinases has not been determined. Furthermore, the effect of US28 phosphorylation and arrestin binding on ligand dependent US28 signaling pathways such as pro-migratory signaling to FAK and ERK remains to be elucidated. Certainly, the dynamic regulation of US28 signaling in different signaling and cell type contexts deserves further study.

Pharmacological Targeting of US28

To date, only one pharmacological means for inhibiting US28 has been characterized. Casarosa et. al. characterized a nonpetidergic CCR1 antagonist, VUF2274, as an inverse agonist for US28. This study demonstrates that VUF2274 interaction with US28 is able to inhibit the constitutive activation of PLC signaling pathways. Mutagenesis of the receptor indicates that VUF2274 does not interact with the chemokine binding domain, but instead binds to residues in the hydrophilic pocket formed by the 7TM helices (Casarosa et al., 2003). However, VUF2274 appears to have no inhibitory effect on the chemokine-dependent signaling of US28 to FAK, or the production of actin cytoskeletal rearrangements in fibroblasts (Streblow, unpublished observations). Subsequently, Hulshof et. al. performed detailed pharmacological characterization of VUF2274 and related compounds, producing

several drugs with increased selectivity for US28 that are capable of inhibiting the constitutive activation of PLC (Hulshof et al., 2005; Hulshof et al., 2006). The efficacy of these drugs for inhibition of US28 ligand-dependent signaling remains to be determined.

Summary and Conclusions

Recent research has revealed a startling complexity associated with signaling from the HCMV-encoded chemokine receptor US28. US28 is able to respond to both the extracellular chemokine environment and the intracellular complement of G-proteins, signaling molecules and regulatory molecules to produce a wide variety of signaling and cell motility responses. This highly context-specific functionality requires particular attention with respect to pharmacological targeting of US28. It is apparent that a any potential US28 antagonists must be tested for efficacy against a number of US28-dependent signaling pathways as well as in a number of HCMV-susceptible cell types. Furthermore, characterization of the regulatory proteins interacting with US28 in different cell types may provide targets for cell-type specific inhibition of US28 functions. US28 provides an interesting example of a GPCR able to exert ligand- and cell-type specific signaling. Further study of the mechanisms behind this functional selectivity may serve to elucidate more general aspects of GPCR biology.

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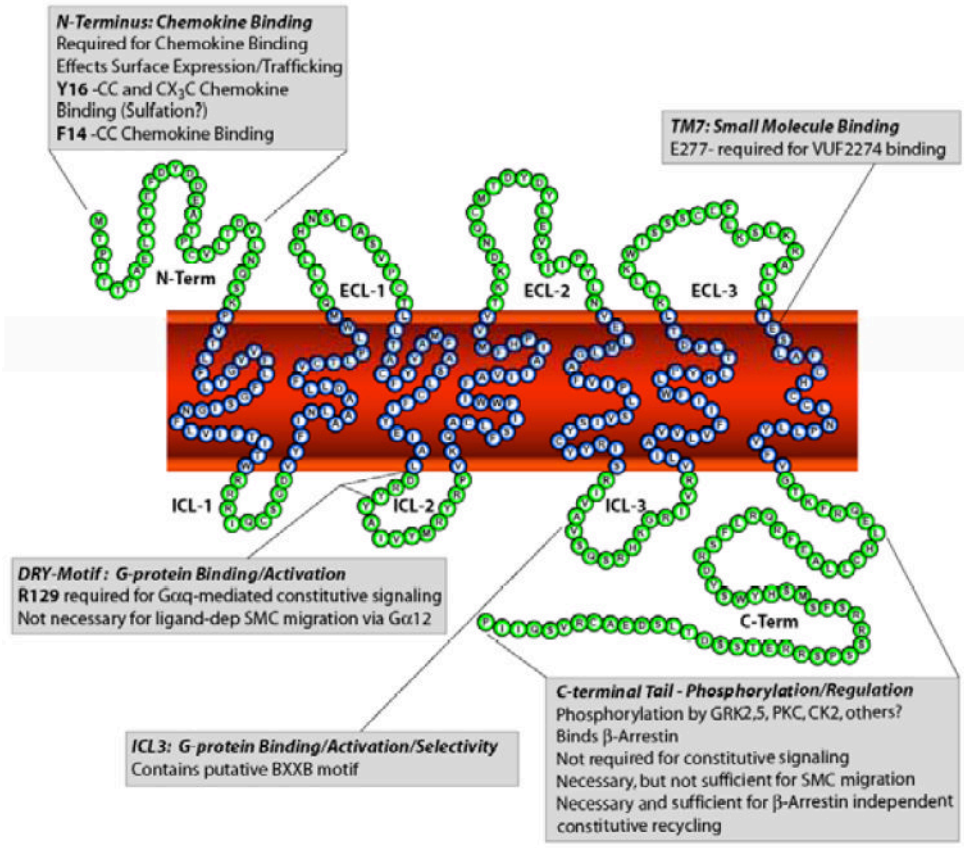

Fig. 1.

Ribbon structure model for US28. Regions of US28 with known or predicted functional significance are indicated by call outs. Non-italic boldface type indicates specific residues of US28. Abbreviations used: Extracellular Loop (ECL), Intracellular Loop (ICL), Amino Terminus (N-Term), Carboxyl Terminus (C-Term), Smooth Muscle Cell (SMC).

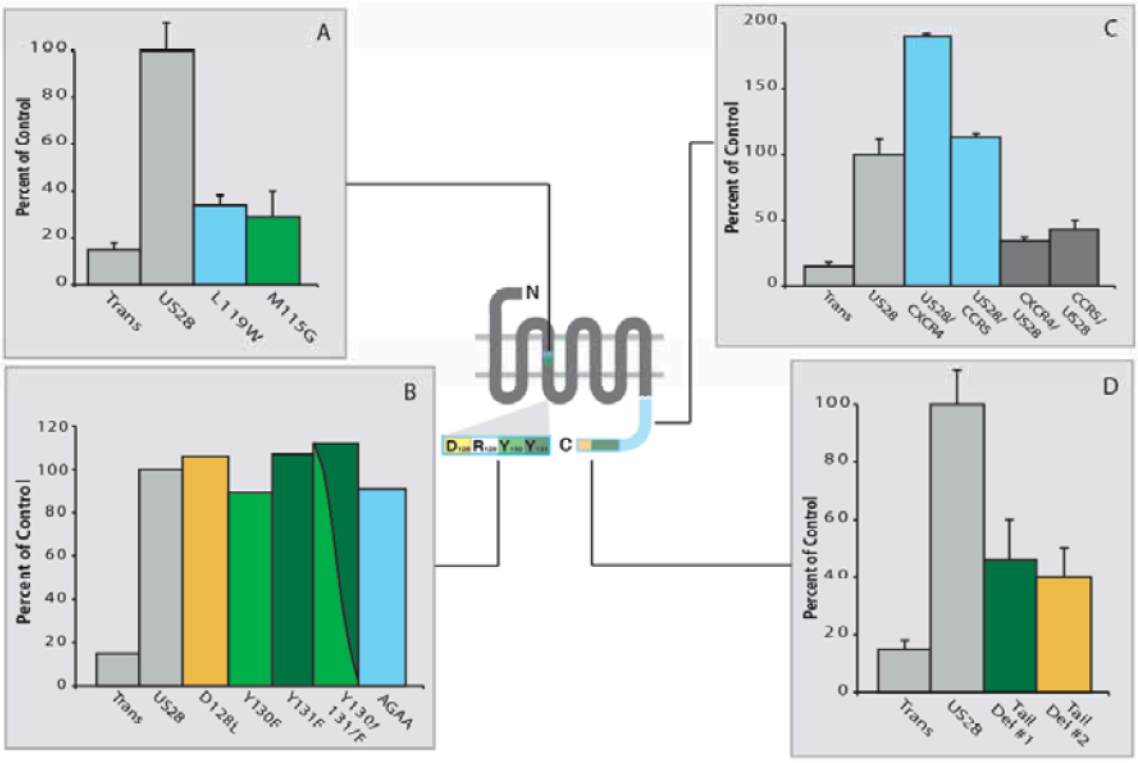

Fig. 2.

Smooth muscle cell (SMC) migration data for several US28 functional domains. All US28 mutants were expressed via tet transactivator inducible adenovirus vector system. 'Trans' controls indicate cultures infected with Ad-Trans transactivator adenovirus only. (A) US28 mutations affecting intrahelical packing of US28 abrogate SMC migration. (B) Mutations in the 'DRY' motif, a putative active domain of US28 have no effect on CC-chemokine mediated SMC migration. In the US28-AGAA mutant the entire DRYY motif was replaced with the indicated AGAA sequence (C) Chimeric proteins in which the C-terminal tail domain of US28 is replaced with the tail domains of CXCR4 and CCR5 can still mediate SMC migration. Chimeric proteins of CXCR4 and CCR5 containing the US28 C-terminal tail are not able to cause SMC migration. (D) Deletion of the C-terminal 10 (Tail Del #2) or 18 (Tail Del #1) amino acids of US28 significantly abrogates SMC migration.